A mint plant '*Indus*'

Field of the present Invention

The present invention relates to a new and distinct mint plant of *Mentha piperita* '*Indus*', selected through screening, field trial and analysis of monoterpene constituents of the essential oil of the germplasm, possessing the characters of producing high amount of menthofuran ranging between 22 to 30% of total oil content, high amount pulegone ranging between 9.0 to 18% of total oil content, with essential oil content ranging between 0.32 to 0.40% of the total oil content.

Background and prior art references of the present Invention

Menthofuran (3,6-dimethyl-4,5,6,7-tetrahydrocoumarone) is one of the major constituent for aroma of the essential oil extracted from the leaves of *Mentha piperita*. Because any other compound has not duplicated the aroma effect, menthofuran is important in the formulation of certain standardized essential oils, such as peppermint oil. However, menthofuran is an expensive compound of limited availability as the plants produce 0 to 6% menthofuran (US Patent PP11,788). Literatures are available for the chemical synthesis of menthofuran to substitute the naturally available menthofuran (US patent 4,240,969) to reduce cost of production. Also the acceptability of synthetic menthofuran is a limiting factor in determining the cost of the essential oil mixture containing synthetic components in aroma industry.

Considering the importance of menthofuran in aroma industry under 'New Millennium Indian Technology Initiative (NMITLI) programme' launched by Council of Scientific and Industrial Research (CSIR), India, during 2001, a systematic approach was undertaken to evaluate the existing germplasm of *M. piperita* at CIMAP and breed for genetic enhancement towards high menthofuran biosynthesis in the essential oil. Systematic breeding experiments to allow open pollination followed by single seed progeny selection by chemotypic

evaluation for enhanced constituent (menthofuran) led to development of this chemotype 'Indus'.

Objects of the present invention

The main object of the present invention is to develop a new and distinct mint plant.

Another main object of the present invention is to develop a novel mint plant through screening, field trial and analysis of monoterpene constituents of the essential oil of the germplasm.

Yet another object of the present invention is to develop a plant producing high amount of menthofuran.

Still another object of the present invention is to develop a plant producing high amount pulegone.

Still another object of the present invention is to develop a plant producing high herbage.

Still another object of the present invention is to develop a mint plant showing resistance against major plant disease conditions like leaf spot, rust, powdery mildew, lepidopteran pest *Spilarctia obliqua*.

Summary of the present invention

The present invention relates to a new and distinct mint plant of *Mentha piperita* '*Indus*', selected through screening, field trial and analysis of monoterpene constituents of the essential oil of the germplasm, possessing the characters of producing high amount of menthofuran ranging between 22 to 30% of total oil content, high amount pulegone ranging between 9.0 to 18% of total oil content, with essential oil content ranging between 0.32 to 0.40% of the total oil content,

Detailed Description of the present invention

Accordingly, the present invention relates to a new and distinct mint plant of *Mentha piperita 'Indus'*, selected through screening, field trial and analysis of monoterpene constituents of the essential oil of the germplasm, possessing the characters of producing high amount of menthofuran ranging between 22 to 30% of total oil content, high amount pulegone ranging between 9.0 to 18% of total oil content, with essential oil content ranging between 0.32 to 0.40% of the total oil content,

A new and distinct mint plant of *Mentha piperita 'Indus'*, selected through screening, field trial and analysis of monoterpene constituents of the essential oil of the germplasm, possessing the following combination of characters:

a. the said plant produces high amount of menthofuran ranging between 22 to 30% of total oil content,

b. the said plant produces high amount pulegone ranging between 9.0 to 18% of total oil content,

c. the said plant produces essential oil content ranging between 0.32 to 0.40% of the total oil content,

d. the said plant produces herbage yield ranging between 200-220 Q per ha,

e. the said plant is of height ranging between 65 to 70 cms, with plant canopy of area ranging between 78-85 cms,

f. the said plant shows resistance against leaf spot, rust, powdery mildew, lepidopteran pest *Spilarctia obliqua*,

g. the said plant has Quadrangular, woody stems, of color purplish green with RHS color code of 59A,

h. the said plant has simple, opposite, and decussate leaves, of dark green color with color code of RHS 137A,

i. the said plant has leaves of chartaceous texture,

j. the said plant's leaves has Glabrous dorsal surface, with hairy on ventral veins,

k. the said plant has leaves of ovate-elliptical shape, with serrate margins,

l. the said plant has leaves with acute-acuminate tip, obtuse base, and broad size,

m. the said plant has leaf with petiole of length ranging between 0.5 to 0.9 cm,

n. the said plant has leaf with of area about 7.91 cm^2,

o. the said plant has leaf with length ranging between 1.2 to 4.3 cm,

p. the said plant has leaf width ranging between 0.6 to 2.6 cms,

q. the said plant has inflorescence of nature terminal spike,

r. the said plant has flowers of following traits:

    i. arranged in whorls,

    ii. smooth pedicel,

    iii. green color pedicel with RHS code 137B,

    iv. calyx is glabrous, tubular, 5-lobed, margin ciliated, yellow green with RHS color code of 146C,

    v. corolla is purple, tubular, 4-lobed, with subsequeal lobes,

    vi. white flowers,

    vii. anthers are four in number, exserted, grayed-red with RHS code of 181A,

    viii. stigma is bifid.

s. the said plant is able to produce higher herbage, menthofuran and pulegone yield per unit area as compared to other existing improved varieties,

t. the said plant produce high menthofuran when harvested 75 days after planting and 115 days after planting,

u. the said plant produce high pulegone when harvested 75 days after planting,

v. the said plant is able to produce higher pulegone and menthofuran due to up regulation and thus has the potential to isolate regulatory factors for monoterpene metabolism, and

w. the said plant has distinct molecular profile by random amplified polymorphic DNA (RAPD) using 20 random primers (OPA) distinguishing the plant from the other existing varieties.

The present invention is related to the development of a novel high menthofuran and pulegone producing chemotype which can also yield high amount of pulegone through proper harvest management. The plant chemotype was obtained through screening of the open pollinated seed progenies of the variety 'Kukrail'. The invention is further related to the plant producing more herb yield leading to higher production of essential oil per unit area compared to the seed parent variety. The selected plant possesses the property of accumulating more menthofuran and pulegone at specific developmental stages and hence proper management prior to processing can yield high amount of these important phytochemicals for industrial use. This plant is unique and clearly distinct from all other existing varieties of *Mentha piperita*. The new plant type 'Indus' can be propagated vegetatively through suckers and runners for commercial cultivation.

'Kukrail' is a released variety of CIMAP which is maintained along with the germplasm of CIMAP in the field systematically every year. Every year in the month of October, the twigs are planted in small sized plots (3m X 3m) for generation of enough planting material for planting in the main field during the month of January. Open pollinated seeds are collected from different genotypes every year and analyzed for monoterpene constituents in the essential oil.

CIMAP/MP20 is such a genotype selected from open pollinated seed progenies of the variety 'Kukrail'. *Mentha piperita* is propagated vegetatively through runners. With the NMITLI initiative the runners generated from the seed plots were planted in 5m X 5m plots during the month of January 2001, following normal agronomic practices with the objective to screen genotypes rich in menthofuran in the essential oil. Replicated samples from each genotypes were taken from the field by planting multiplied runners in the month of January, 2001, 2002 and 2003 for 3 consecutive years in RBD fashion and different growth and yield characteristics were recorded (Table 1). For field trials the replicated plots were prepared by adding only FYM 1.5 ton per ha. The three-year averages of herb yield, essential oil yield and the variations in major essential oil components are detailed below for the genotype CIMAP/MP20 compared to the CIMAP released varieties of *Mentha piperita* 'Kukrail', 'Tushar', 'Pranjal'. 'Pranjal' bears the patent no PP14,090.

Table 1: Comparative herbage, oil, menthofuran and pulegone yield of *Mentha piperita* genotypes.

Genotypes	CIMAP/MP20	Kukrail	Tushar	Pranjal
Oil content (%)	0.35	0.40	0.63	0.55
Herrbage yield (Quintal/ hectare)	206.8	123.8	190.5	123.8
Oil yield (Litre per hectare)	72.41	49.52	119.04	68.10
Menthofuran content(%)	27.24	8.727	9.648	8.385
Calculated Menthofuran yield (Litre per hectare)	19.72	4.32	11.484	5.710
Pulegone(%)	15.405	3.032	2.355	2.783
Calculated Pulegone yield (Litre per hectare)	11.155	1.501	2.80	1.89

If menthofuran is aimed the genotype can yield highest amount of natural menthofuran than any other variety released and reported which is the case for pulegone also.

Oil extraction and GLC analysis

Oil samples from the field grown plants were extracted by hydrodistillation using Clevenger's apparatus and weighed to record the yield. Over ground shoot samples were collected from the whole plant selected randomly from the middle of the row of each replicated plots at different days after planting (35. 55, 75, 95, 115 days after planting). Shoots collected from individual genotypes were bulked for each treatment plot and essential oil was distilled from all the replicates taking 500g of bulked shoots containing leaves. The final analysis of all the essential oil samples was accomplished on Varian CX-3400 using a 30m X 0.25mm (0.25 □) Supelcowax-10 column. The injector and detector temperature were maintained at 200 and 225°C respectively, with oven temperature programmed from 60 to 200 °C at the rate of 7 °C min^{-1} increase, with initial and final holds of 2 and 5 minutes respectively. Hydrogen gas was used as carrier at the rate of 1ml min^{-1} and 0.1 □l of sample was injected with a split ratio of 1:50. Data were processed in the electronic integrator Varian 4400 and the identification was based on retention time of authentic samples of l-menthol (Takasago, Japan) and retention indices calculations (Jennings W & Shibamoto T (1980) Qualitative analysis of flavour and fragrance volatile by capillary GC, Academic Press Inc., New York.).

Table 2: Variation in major essential oil components of *Mentha piperita* genotype CIMAP/MP20 at different stages of growth.

	35 DAYS	55 DAYS	75 DAYS	95DAYS	115DAYS
Limonene	0.6405	6.5948	5.6849	5.6283	4.484
Menthone	1.4922	48.55	2.0802	3.8298	1.9770
Menthofuan	1.4922	6.2713	23.96	3.4181	27.246
Menthol	32.5681	34.138	12.7920	28.2289	14.396
Pulegone	9.6714	0.2421	10.4367	16.8836	15.405

Table 3: Comparative monoterpene component profiles in the essential oil of *M.piperata* P(20), Kukrail, Tushar, Pranjal, 115 days after planting.

	Components	CIMAP/MP20	Kukrail	Tushar	Pranjal
1	α- Pinene	0.432	0.465	0.661	0.707
2	β- Pinene	1.004	0.874	1.256	1.472
3	Sabinene	0.857	1.031	0.786	0.881
4	Myrcene	4.897	0.342	0.342	0.341
5	α- terpinene	0.081	0.244	0.089	0.069
6	Limonene	4.484	2.862	2.656	3.145
7	1,8 Cincole	8.809	4.904	5.112	5.252
8	γ – Terpinene	0.769	0.226	0.244	0.248
9	p-Cymene	0.304	0.309	0.105	0.130
10	3-Octanol	0.133	0.127	0.258	0.322
11	Menthone	1.970	21.292	28.248	28.339
12	Menthofuran	27.246	8.727	9.648	8.385
13	Iso menthone	0.556	3.963	4.410	4.084
14	Menthyl acetate	2.323	7.857	4.768	3.799
15	Neo menthol	4.757	3.870	2.897	3.288
16	□- Caryophyllene	0.661	0.452	0.088	0.056
17	Pulegone	15.405	3.032	2.355	2.783
18	Menthol	14.396	28.840	26.818	26.147
19	Piperitone	1.536	2.331	1.056	1.232
20	Carvone	0.606	0.376	0.585	0.796

The genotype CIMAP/MP20 has a characteristic oil profile which expresses differentially at different stages of growth. The menthofuran content was found to be higher at 75 days stage which decreased during 95 days and again increased during harvesting time 115 days. Corresponding menthol content in the essential oil content was found to be negatively correlated to the menthofuran content at corresponding stages of growth.

Pulegone content increased after 75 days and was stabilized after 95 to 115 days (Table 2). The comparative monoterpene profiles for different components is presented in Table 3 during harvesting time (115 days stage).

Trichome analysis of the genotype CIMAP/MP20

Monoterpenes are known to be cytotoxic to plant tissues, inhibiting respiration and photosynthesis by drastically affecting the mitochondria, golgi bodies etc and decreasing cell membrane permeability (Brown J T, Hegarty P K & Charlwood B V (1987) The toxicity of monoterpenes to plant cell cultures. Plant Science 48:195-201.). Monoterpenes are either sequestered in the plants in specialized structures like glandular hairs in *Pelargonium* (Brown J T & Charlwood B V (1986) Differentiation and monoterpene biosynthesis in plant cell cultures. In; Morris P, Scragg A, Stafford A and Fowler M (eds) Secondary Metabolism in Plant Cell Cultures. Cambridge University Press, Cambridge, 1986, p.68.), trichomes in *Mentha* or stored in the form of non-toxic glycoside derivatives in vacuoles e.g. *Rosa* spp.

So the number of trichomes at different developmental stages of the genotype CIMAP/MP20 and its variation in different leaves (both the upper and lower surface) situated at different level (0 level: leaf at the tip, 1 level : next leaf down to 0 level, 2 level : next leaf down to 1 level, 3 level : next leaf down to 2 level, 4 level : next leaf down to 3 level,) were characterized and finally all the trichome at different levels of the leaves were averaged, calculated per centimeter square leaf area. A peak trichome density was observed from 75 to 95 days in all the leaves at different levels except the leaf at the tip. At 0 level the leaves are at active developmental stage which may be cause for steady rate for trichome formation (Table 4).

Table 4. Trichome density(Trichomes/ cm^2) in the leaves of the genotype CIMAP/MP20 at different developmental stages.

Levels	35 days stage	55 days stage	75 days stage	95 days stage	115 days stage
0	1416	2816	5499	5392	5400
1	1223	2592	4373	5112	3106
2	982	1349	2443	2464	2392
3	875	1168	1813	2080	1668
4	610	752	1824	1205	1477

Taxonomic description of the peppermint plant CIMAP/MP20 are as given below:

1. Genus	: ***Mentha***
2. Species	: ***piperita***
3. Family	: Lamiaceae
4. Common name	: Peppermint
5. Plant height	: 65-70cm
6. Plant canopy	: 80-84cm
7. Growth habit	: Herbaceous, erect and branched
8. Stem	:Quadrangular, woody, purplish green (59A)
9. Leaf	:Simple, opposite, decussate
Colour	Dark green (137A)
Texture	Chartaceous
Surface	Glabrous dorsal surface, hairy on ventral veins
Shape	ovate-elliptical
Margin	serrate
Tip	acute-acuminate
Base	Obtuse
Size	broad
Petiole length	0.5cm-0.9cm
Area	7.91cm^2
Length	1.2cm-4.3cm
Width	0.6cm-2.6cm
10. Leaf : stem ratio	:1.06

11. Inflorescence	:Terminal spike
12.Flowers	:Arranged in whorls
Pedicel	smooth, green (137B)
Calyx	Glabrous, tubular, 5-lobed, margin ciliated, yellowgreen(146C)
Corolla	purple, tubular,4-lobed,lobes subequeal, white
Anthers	four,exserted, Grayed-red (181A)
Stigma	Bifid

The colour codes are in accordance with the "RHS colour chart published by the Royal Horticultural Society, 80 Vincent Square, London SW1P 2PE,1995. The genotype CIMAP/MP20 was named and referred as 'Indus' in this specification.

DNA isolation and PCR amplification reactions

DNA was isolated from leaf tissue essentially according to the protocol described previously (Khanuja S P S, Shasany A K, Darokar M P & Sushil Kumar (1999) Rapid Isolation of PCR Amplifiable DNA from the Dry and Fresh Samples of Plants Producing Large Amounts of Secondary Metabolites and Essential oils by Modified CTAB Procedure. Plant Molecular Biology Reporter 17: 74.) and pooled DNA (equal amount from 20 individual plants of a genotype in a field) constituted the samples for polymerase chain reactions (PCRs) which were carried out in 25 μl volume .

A reaction tube contained 25 ng of DNA, 0.2 unit of Taq DNA polymerase , 100 μM each of dNTPs , 1.5 mM $MgCl_2$ and 5 p mol of decanucleotide primers. The amplifications were carried out using the DNA Engine thermal cycler (MJ Research, USA) following the protocol of Khanuja et al. (Khanuja S P S, Shasany A K, Srivastava A & Sushil Kumar (2000). Assessment of genetic relationships in *Mentha* species. Euphytica 111: 121-125.). The amplified products were loaded in 1.2% agarose gel containing 0.5 μg ml^{-1} of ethidium bromide and photographed by Polaroid system. Twenty decamer primers

procured from Operon Technologies, USA (OPA) were used to detect polymorphism in the selected genotype. The similarity matrix obtained after multivariant analysis using Nei and Li's coefficient (Nei, N. & W. Li, 1979.

Brief description of the accompanying drawings

Figure 1 shows cluster analysis for the chemotype "Indus" compared to 'Kukrail', Tushar', and 'Pranjal'.

Figure 2 show RAPD profile of the *Mentha piperita* genotype CIMPA/MP20

Figure 3 shows A twig of CIMAP/MP20

Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. (USA) 76: 5269-5273.) is shown in Table 5. These similarity coefficients were used to generate a tree for cluster analysis using UPGMA method (Figure 1) which shows the distinctiveness of the genotype CIMAP/MP20. The genotype CIMAP/MP20 was 54.1%, 50.2% and 51.1% different with the varieties 'Kukrail', 'Tushar' and 'Pranjal' respectively establishing the uniqueness of the genotype. These primers were also used to develop a unique RAPD profile of the chemotype 'Indus' (Figure 2).

Table 5: Similarity between the genotypes compared through RAPD profile analysis.

	Kukrail	Tushar	Pranjal	CIMAP/MP20
Kukrail	1.000			
Tushar	0.960	1.000		
Pranjal	0.950	0.960	1.000	
CIMAP/MP20	0.459	0498	0.489	1.000

Uniformity and stability

Like any other *Mentha piperita* genotype this genotype is also planted vegetatively through runners and suckers. No variation of any kind was observed in this genotype for the last 3 years of trial maintaining the quality of oil and phenotype. The RAPD analysis of random plant samples in different

years of trial also did not show any variation in profiles for this genotype indicating the stability of this genotype.

Disease and pest resistance

The incidence of lepidopteran pest *Spilarctia obliqua* and fungus mint rust (*Puccinia sps*), leaf spot and mildew were not detected in the field continuously for 3 years in the genotype CIMAP/MP20.

Metabolic regulation of menthofuran biosynthesis

The genotype CIMAP/MP20 was rich in pulegone and menthofuran. In the biosynthetic pathway Geranyl pyrophosphate is converted to limonene which in turn is transformed into isopiperitenol, followed by pulegone. Pulegone is converted to menthone followed by menthol. Menthol and Menthone are the main constituents of the essential oil of *Mentha piperita*. In one branch of the pathway pulegone is converted to menthofuran. In this genotype the monoterpene pulegone is biosynthesized at an accelerated rate and the reaction favour more towards the menthofuran synthesis than menthol. In the initial stage (up to 55 days stage) of growth of this genotype the reaction favours for the production of menthol at a reduced rate from pulegone with less accumulation of pulegone and menthfuran. But at later stage as the plant matures the reaction favours accumulation of more menthofuran and pulegone and instead the biosynthesis of menthol decreases. This indicate the role of regulatory proteins in the monoterpene metabolism and the importance of this genotype for the isolation of such protein for future modification of metabolic pathway modification.